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(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ERRINGTON, Jeffery [GB/GB]; 6 Sandfield Road, Headington, Oxford OX3 7RG (GB). WU, Ling, Juan [CN/GB]; 4 Ablett Close, Oxford OX4 1XH (GB).

(74) Agent: PENNANT, Pyers; Stevens Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

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(54) Title: BACILLUS STRAIN AND ANTIBIOTIC SCREENING METHOD

(57) Abstract

A *Bacillus* strain has a chromosome with the following modifications: a mutation of a spoIIIE gene which blocks transfer of the prespore chromosome; a mutation which prevents loss of SpoOJ function from blocking sporulation; a first reporter gene dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to increased trapping in the prespore; and a second reporter gene having a promoter which is dependent on σ^F factor and where impaired SpoOJ function leads to reduced trapping in the prespore. The strain is useful in a method of screening for putative antibiotics.

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BACILLUS STRAIN AND ANTIBIOTIC SCREENING METHOD

Background

We previously showed that the SpoIIIE protein of *Bacillus subtilis is* required for the transfer of the prespore chromosome through the asymmetric division septum that separates the prespore from its larger mother cell *(1,2). spoIIIE* mutations lead to a block in sporulation, leaving the prespore with only part of a chromosome, the remainder of the chromosome being trapped in the mother cell compartment. Further analysis of *spoIIIE* mutants has revealed that the small segment of DNA that is trapped in the prespore is a fairly specific one, centred close to the origin of DNA replication, *oriC* (1,3). This implied the existence of a mechanism which imposes a specific orientation on the chromosome destined for the prespore before septation. We have recently shown that the *spoOJ* gene is required to specify this orientation (4). However, specificity is not completely lost in *spoOJ* mutants (3), so it appears that there must be at least one secondary mechanism working to determine chromosome orientation at the onset of sporulation. The assay for inhibitors of SpoOJ function described below exploits some unexpected features of this change of specificity.

The *spoOJ* gene is highly conserved in a wide range of bacteria (5) and it is related to a family of proteins required for accurate partitioning of low-copy-number plasmids found in many diverse bacteria (6,7). Our recent results strongly suggest that SpoOJ protein has a direct role in segregation of sister chromosomes during both growth and sporulation (10). However, the gene is not essential for vegetative growth, although chromosome partitioning is partially impaired (8). Most likely, this is because of the presence of a secondary partitioning system in this

organism, perhaps the same one that we have detected in the experiments mentioned above. Nevertheless, there is at least one report of a chromosomal *spoOJ*-like gene being essential (9), consistent with the vital importance of chromosome partitioning mechanisms for bacterial viability. Thus, the *spoOJ* family of proteins may be good targets for antimicrobial agents .

The Invention

The effects of *spoOJ* mutations on prespore chromosome orientation, and the ability to detect this by use of a *spoIIIE* mutant background, provides the potential for a very specific whole-cell assay for inhibitors of SpoOJ function. The presence of any given segment of chromosomal DNA in the prespore can be detected by use of a reporter gene controlled by a transcription factor, σ^F, which is activated only in the small prespore compartment (a process that is not affected by perturbations in chromosome partitioning).

Thus the invention provides in one aspect a *Bacillus* strain having a chromosome with the following modifications:

a) a mutation of a *spoIIIE* gene which blocks transfer of the prespore chromosome,

b) a mutation which prevents loss of SpoOJ function from blocking sporulation, together with

c) a first reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to increased trapping and hence to increased expression in the prespore, and/or

d) a second reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to reduced trapping and hence to reduced expression in the prespore.

In another aspect the invention provides a method of determining whether an agent inhibits SpoOJ function in *Bacillus* species, which method comprises inducing the *Bacillus* strain as described to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and/or the second reporter gene.

In another aspect the invention provides a method which comprises inducing the *Bacillus* strain as described to sporulate in the presence of an agent, observing expression of the first and/or second reporter gene and thereby determining that the agent inhibits SpoOJ function in the *Bacillus* species, and using the agent as an antibiotic to kill bacteria.

In yet another aspect the invention provides a method of killing bacteria which method comprises contacting the bacteria with a substance which inhibits SpoOJ function in *Bacillus* species.

Preferably both the first reporter gene c) and the second reporter gene d) are present in the *Bacillus* strain of the invention. Preferably each of the first reporter gene c) and the second reporter gene d) is fused to a σ^F dependent gpr promoter. Preferably each of the first reporter gene c) and the second reporter gene d) expresses a different detectable enzyme.

In the method of the invention, expression of the first and second reporter genes is preferably observed by monitoring changes in the levels of or the ratio of their products. Preferably the first and second reporter genes are expressed as enzymes whose activities are observed by any convenient means e.g. fluorimetry or spectrophotometry. Preferably the *Bacillus* strain is induced to sporulate and is contacted, just prior to asymmetric cell division, with the agent being investigated. The method can conveniently be performed as a screening test for putative antimicrobial agents.

Any *Bacillus* species may be used that is capable of

sporulating under suitable conditions and for which genetic constructions can be made. *B subtilis* is conveniently accessible and well characterised and is preferred.

Experiments with reporter genes placed at many locations in the chromosome have shown that in a *spoIIIE* mutant a fairly specific segment of DNA is trapped in the prespore compartment (1, 3). Loss of the *spoOJ* gene results in a change in the specificity of the segment of DNA that is trapped, which leads to characteristic changes in the levels of expression of σ^F-dependent reporter genes placed at different chromosomal locations. Figure 1 shows the effect of a *spoOJ* mutation on expression of a σ^F-dependent reporter gene (*gpr-lacZ*) placed at different chromosomal locations in a *spoIIIE36* background. To control for day to day variation in absolute β-galactosidase levels, each experiment was done with a control strain comprising the reporter at the same location in a *spoIIIE+* background. Each activity is expressed relative to that of the corresponding control. Thus, for example when a *spoOJ* mutation is combined with the *spoIIIE* mutation, expression of the reporter gene increases at the *sigL* location but decreases at the *amyE* location. Loss of SpoOJ function can therefore be detected by the large change that characteristically occurs in the levels of the products of the two reporters.

A preferred assay strain (e.g. 1238) contains several genetic modifications. First, a point mutation in the *spoIIIE* gene to block transfer of the prespore chromosome. The *spoIIIE36* mutation is a convenient, well characterised, example of the appropriate type (1, 2). Second, a *soj* mutation, to prevent the loss of SpoOJ function from blocking sporulation (8). Any mutation abolishing *soj* function without unduly affecting expression of the adjacent *spoOJ* gene; such as the large in-frame deletion constructed by Ireton *et al* (8) would be appropriate. Third, a reporter gene, *lacZ,* fused to the σ^F-dependent *gpr* promoter and placed at the *sigL* location, where impaired SpoOJ function leads to increased

trapping in the prespore and thus increased synthesis of the reporter gene product, β-galactosidase. Fourth, a second reporter gene, similar to the first but placed at the *amyE* location, where trapping and thus expression is reduced when SpoOJ function is impaired, and based on the *gus* gene (also called *uidA*), encoding, β-glucuronidase. In the absence of inhibitors of SpoOJ, sporulating cells of this strain produce considerably more β-glucuronidase than β-galactosidase. Inhibitors of SpoOJ would result in a dramatic change in the ratio, with decreased β-glucuronidase and increased β-galactosidase. Non-specific inhibitors affecting cell viability, ability to sporulate, activation of σ^F, or one or other of the reporter enzymes, would not produce this characteristic change.

The assay could be readily adapted to run on a high throughput basis, so as to enable the screening of large libraries of compounds. Strain 1238 would be grown in large batch culture in a hydrolysed casein growth medium and induced to sporulate by harvesting and resuspension in a starvation medium, according to standard practice (11,12). Samples of the sporulating culture would then be dispensed into the individual wells of microtitre plates containing potential inhibitors. After an appropriate period of incubation, to allow activation of σ^F and expression of the two reporter genes, the cells would be lysed and assayed simultaneously for the two enzyme products. In the case of β-galactosidase and β-glucuronidase, there are a range of substrates available for assaying the specific enzyme activities. These can give fluorescent, chemiluminescent or coloured products, which could be measured either on a continuous or a fixed time basis, using automated plate readers. Potential inhibitors could be reinvestigated in more detail using other assay methods or bacterial strains with different combinations of reporter genes. They should also produce characteristic changes in the microscopic appearance of sporulating cells (4).

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CLAIMS

1. A *Bacillus* strain having a chromosome with the following modifications:

a) a mutation of a *spoIIIE* gene which blocks transfer of the prespore chromosome,

b) a mutation which prevents loss of SpoOJ function from blocking sporulation, together with

c) a first reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to increased trapping and hence to increased expression in the prespore, and/or

d) a second reporter gene having a promoter which is dependent on σ^F factor and placed at a location where impaired SpoOJ function leads to reduced trapping and hence to reduced expression in the prespore.

2. A *Bacillus* strain as claimed in claim 1, wherein b) is a *soj* mutation.

3. A *Bacillus* strain as claimed in claim 1 or claim 2, wherein each of the first reporter gene c) and the second reporter gene d) is fused to a σ^F-dependent factor *gpr* promoter.

4. A *Bacillus* strain as claimed in any one of claims 1 to 3, wherein each of the first reporter gene c) and the second reporter gene d) expresses a different detectable enzyme.

5. A method of determining whether an agent inhibits SpoOJ function in *Bacillus* species, which method comprises inducing the *Bacillus* strain of any one of claims 1 to 4 to divide asymmetrically, as during sporulation, in the presence of the agent, and observing expression of the first and/or the second reporter gene.

6. A method as claimed in claim 5, wherein expression of the first and second reporter genes is observed by monitoring the levels of their expression products.

7. A method as claimed in claim 6, wherein the first and second reporter genes are expressed as enzymes whose activities are observed by fluorimetry or spectrophotometry.

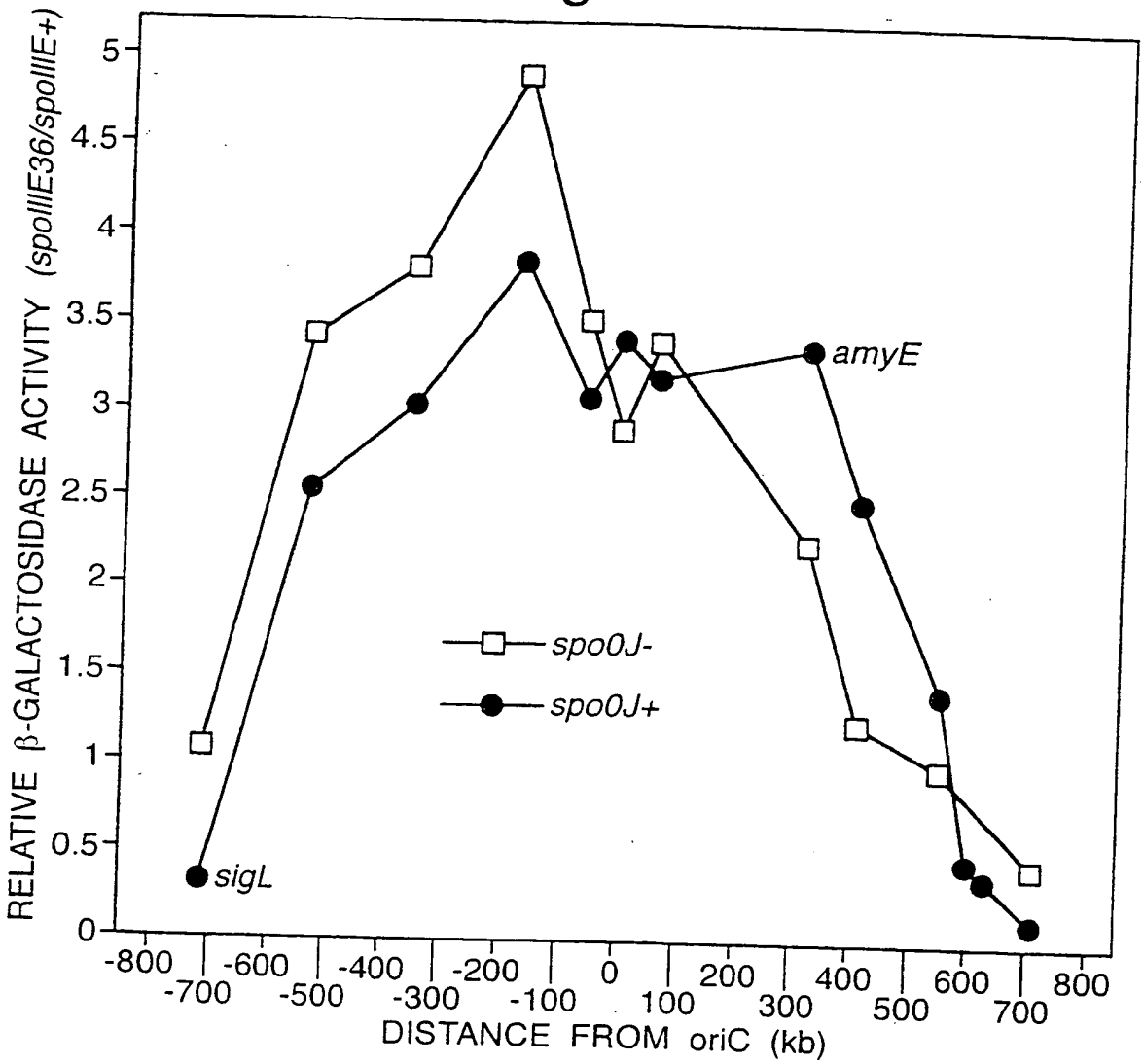
8. A method as claimed in any one of claims 5 to 7, wherein the *Bacillus* strain is induced to sporulate and is contacted, just prior to asymmetric cell division with the agent.

9. A method as claimed in any one of claims 5 to 8, performed as a screening test for putative antimicrobial agents.

10. A method which comprises inducing the *Bacillus* strain of any one of claims 1 to 4 to sporulate in the presence of an agent, observing expression of the first and/or second reporter gene and thereby determining that the agent inhibits SpoOJ function in the *Bacillus* species, and using the agent as an antibiotic to kill bacteria.

11. A method of killing bacteria which method comprises contacting the bacteria with a substance which inhibits SpoOJ function in *Bacillus* species.

Fig.1.


RELATIVE β-GALACTOSIDASE ACTIVITY (spoIIIE36/spoIIIE+)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
-800
-700
-600
-500
-400
-300
-200
-100
0
100
200
300
400
500
600
700
800
DISTANCE FROM oriC (kb)
spo0J-
spo0J+
amyE
sigL

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/03401

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/18 C12N15/65 //C12N1/21,C07K14/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PARTRIDGE S R ET AL: "THE IMPORTANCE OF MORPHOLOGICAL EVENTS AND INTERCELLULAR INTERACTIONS IN THE REGULATION OF PRESPORE-SPECIFIC GENE EXPRESSION DURING SPORULATION IN BACILLUS SUBTILIS" MOLECULAR MICROBIOLOGY, vol. 8, no. 5, 1993, OXFORD GB, pages 945-955, XP002060307 cited in the application see page 952, column 2, paragraph 2 - page 953 --- -/--	1,3-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
6 April 1998	21.04.98

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DONGXU SUN ET AL: "EFFECT OF CHROMOSOME LOCATION OF BACILLUS SUBTILIS FORESPORE GENES ON THEIR SPO GENE DEPENDENCE AND TRANSCRIPTION BY EOF: IDENTIFICATION OF FEATURES OF GOOD EOF-DEPENDENT PROMOTERS" JOURNAL OF BACTERIOLOGY, vol. 173, no. 24, December 1991, WASHINGTON US, pages 7867-7874, XP000601088 see page 7868 - page 7869; table 1 ---	1-7
A	IRETON K ET AL: "SpoOJ is required for normal chromosome segregation as well as the initiation of sporulation in Bacillus subtilis" JOURNAL OF BACTERIOLOGY, vol. 176, no. 17, 1994, WASHINGTON US, pages 5320-5329, XP002060308 cited in the application see the whole document ---	1,2,5
A	SHARPE M E ET AL: "The Bacillus subtilis soj-spoOJ locus is required for a centromere-like function involved in prespore chromosome partitioning" MOLECULAR MICROBIOLOGY, vol. 21, no. 3, 1996, OXFORD GB, pages 501-509, XP002060309 cited in the application see the whole document ---	1,2,5
P,X	WO 97 00325 A (ISIS INNOVATION) 3 January 1997 see the whole document -----	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 97/03401

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 11
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Lack of technical disclosure (Art. 6, PCT)

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

[redacted] al Application No
PCT/GB 97/03401

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9700325 A	03-01-97	NONE	